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Settlement and Metamorphosis of *Capitella* Larvae Induced by Juvenile Hormone-Active Compounds Is Mediated by Protein Kinase C and Ion Channels

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Abstract. The signal transduction pathway by which juvenile hormone-active compounds induce settlement and metamorphosis of metatrochophore larvae of the polychaete annelid *Capitella* sp. I was investigated. The known protein kinase C (PKC) activator phorbol-12,13-dibutyrate was an active inducer of settlement and metamorphosis, whereas H-7, an inhibitor of PKC, inhibited settlement and metamorphosis in response to juvenile hormone III (JH III). JH III and methyl farnesoate (MF) also directly activated, *in vitro*, both a PKC-like enzyme present in *Capitella* homogenates and PKC purified from rat brain. In addition, binding studies using the fluorescent PKC inhibitor RIM-1 revealed the presence of a PKC-like enzyme in intact *Capitella* larvae and juveniles. Settlement and metamorphosis of the larvae was also stimulated by membrane-depolarizing concentrations of KCl. This response to KCl was inhibited by tetraethylammonium. The potassium channel blocker 4-aminopyridine induced settlement and metamorphosis, whereas settlement and metamorphosis in response to JH III was inhibited by the potassium channel ionophore nigericin. Settlement and metamorphosis induced by JH III was inhibited by the calcium channel blockers Ni^{2+}, Zn^{2+}, and verapamil, whereas settlement and metamorphosis was induced by the calcium ionophore A23187. These results suggest that in mediating this response, juvenile hormones may cause activation of PKC, leading to subsequent modulation of potassium and calcium channels.

Introduction

The chemoreception by marine invertebrate larvae of chemical "cues" that are present in the ocean environment and induce settlement and metamorphosis is important for the recognition of habitats that favor growth and reproduction (Chia and Rice, 1978; Rittschof and Bonaventura, 1986; Scheuer, 1990). These settlement signals appear to be specific for different species, as evidenced by findings that larvae of the abalone *Haliotis rufescens* respond to specific chemicals in red algae (Morse *et al.*, 1984), larvae of the nudibranch *Phestilla sibogae* respond to chemicals in corals (Hadfield, 1978, 1984), larvae of the polychaete annelid *Phragmatopoma californica* respond to chemicals present in the burrows of adult worms (Pawlik, 1988, 1990; Jensen and Morse, 1990), and larvae of the sand dollars *Dendraster excentricus* (Burke, 1984) and *Echinarachnius parma* (Pearce and Scheibling, 1990) respond to chemicals produced by adult sand dollars.

In previous studies, we have found that juvenile hormones (JH), which are known morphogens that regulate reproduction and development of insects and crustaceans (Laufer and Borst, 1983, 1988; Laufer *et al.*, 1987), as well as chemicals with juvenile hormone activity in insect cuticle bioassays, are able to induce settlement and metamorphosis of metatrochophore larvae of the polychaete annelid *Capitella* sp. I (Biggers and Laufer, 1992, 1996), which is a subspecies member of the *Capitella* polychaete complex (Grassle and Grassle, 1976). In nature, larvae of *Capitella* sp. I are stimulated to settle and metamorphose (Fig. 1) when they come into contact with chemical inducers present in sediments (Butman *et al.*, 1988), although the identity of these chemicals remains in debate (Cuomo, 1985; Dubilier, 1987), they appear to have JH-activity (Biggers, 1994).

We have now investigated the signal transduction process through which the *Capitella* larvae respond to JH-active compounds. Our results presented in this paper indicate that JH-active compounds stimulate settlement and metamor-

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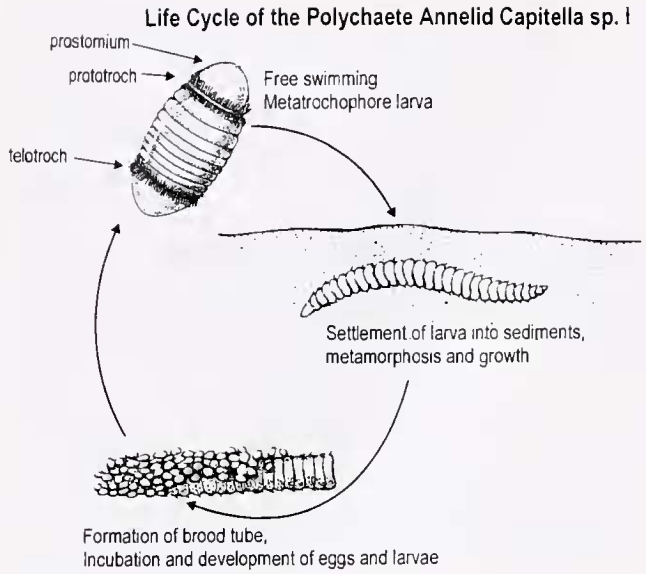

Figure 1. Diagram of the life cycle and metamorphosis of *Capitella* sp. I larvae. In parental brood tubes, trochophore larvae develop into segmented metatrochophore larvae. After hatching and release from the brood tubes, in response to chemical settlement cues, swimming metatrochophore larvae settle and metamorphose into juvenile worms by losing their cilia needed for swimming and developing capillary setae and hooded hooks necessary for crawling through sediments.

phosis of these larvae through the activation of protein kinase C (PKC) and subsequent modulation of ion channels.

Materials and Methods

Capitella *larval settlement bioassays*

Stock cultures of *Capitella* sp. I were maintained at 18°C in large plastic containers containing artificial seawater (Utikem Co.) and washed sea sand (Fisher Scientific) and were fed Tetramin fish food flakes. Brood tubes containing adult females along with their developing eggs and larvae were then separated from the cultures and placed into 60-mm glass petri dishes containing seawater. The dishes were checked daily for hatched, swimming metatrochophore larvae to be used for bioassays. All test chemicals, except for methyl farnesoate (MF) which was synthesized in our laboratory, were purchased from Sigma Chem. Co. Stock solutions of juvenile hormone III (JH III), MF, phorbol-12,13 dibutyrate (PDBU), 1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine (H-7), arachidonic acid, elaidic acid, verapamil, 4-aminopyridine, and nigericin were prepared in 95% ethanol. Stock solutions of KCl, $NiCl_2$, $ZnCl_2$, and tetraethylammonium chloride (TEA) were prepared in distilled water. Settlement and metamorphosis bioassays were conducted at 18°C using 60-mm glass petri dishes that were pre-baked at 250°C to remove contaminants (Biggers and Laufer, 1996). For the assays, 10 to 100 μl of the test chemical stock solutions were added by micropipet into petri dishes containing 10 metatrochophore larvae less than 1 day old (1 day post-release), and 10 ml of artificial seawater. The dishes were then observed. After 1 h, the amount of settlement and metamorphosis was assessed by placing each dish under a dissecting microscope and counting the number of settled larvae crawling on the bottom of the dish. Metamorphosis after 1 hour was also more critically assessed by using a compound microscope and noting the loss of cilia, elongation, and development of capillary setae.

Protein kinase C assays

Assays for the presence of protein kinase C were carried out essentially as described by Yasuda *et al.* (1990), by measuring phosphorylation of an 11-residue synthetic peptide from myelin basic protein (MBP_{4-14}). This method is specific for measurement of PKC, and permits selective measurement in crude tissue preparations. The PKC assay was first standardized using purified rat brain PKC (calcium and phospholipid dependent) from Calbiochem Co. Phosphorylation of MBP_{4-14} was measured using 2–10 ng of enzyme, and over a time period of 30 min. Reactions were carried out in plastic Eppendorf tubes, with 50-μl reaction mixtures containing 20 m*M* Tris/HCl, pH 7.5, 5 m*M* magnesium acetate, 100 μM $CaCl_2$, 25 μM MBP_{4-14} (Sigma Chem. Co.), 50 ng diolein and 500 ng phosphatidylserine, 2–10 ng of rat brain PKC, and 10 μM ATP (containing $5–6 \times 10^5$ CPM gamma ^{32}P-ATP). Reactions were started by addition of enzyme, and incubations were carried out at 30°C for up to 30 min. The reactions were then stopped by placing the tubes on ice and spotting the reaction mixtures onto P-81 anion exchange paper discs (Whatman Co.), which were then immediately immersed in 75 m*M* H_3PO_4, and washed eight times in 100 ml of the same solution. The filter discs were placed in Ecolume cocktail for analysis with a scintillation counter. Other PKC assays using rat brain PKC were carried out in the same manner, except with the replacement of diolein and phosphatidylserine with test chemicals as indicated.

PKC activity in *Capitella* larval homogenates was determined in the same manner, except with the inclusion of the larval homogenates (20–100 μg protein) instead of the rat brain PKC. *Capitella* larvae were collected within 1 day of release from the brood tubes, and were frozen at −20°C. After thawing, the larvae were centrifuged at 5000 rpm for 1 min in a microfuge, the seawater was discarded, and larval homogenates were prepared by homogenizing 1000 larvae in 1 ml 20 m*M* Tris/HCL, pH 7.5, containing 0.5 m*M* phenylmethylsulfonyl fluoride (protease inhibitor) using small glass homogenizers. The homogenates were then centrifuged in a microfuge at 5000 rpm for 1 min to remove large cellular debris, and the PKC activity of the supernatant was assessed as previously described for studies with rat

brain PKC. Protein concentrations of supernatants were determined using a microprotein assay (Sigma Chem. Co.) and a standard curve of increasing concentrations of bovine serum albumin.

Localization of PKC by RIM-1 analysis

Rhodamine-conjugated bisindolylmaleimide (RIM-1) (Calbiochem), a fluorescent PKC inhibitor (Chen and Poenie, 1993), was used to visually locate PKC in *Capitella* larvae and juveniles. Metatrochophore larvae less than 1 day old and 1-day-old juveniles were briefly exposed for 1 min at room temperature to 200 nm RIM-1 in seawater (100 μl) in depression slides covered with aluminum foil. Larvae or juveniles were then fixed in 2% formaldehyde containing seawater for 15 min, transferred into methanol for 15 min to permeabilize the membranes, and then transferred three times (5 min each wash) into 1 ml fresh seawater to rinse away excess unbound RIM-1. Fixed larvae or juveniles were then transferred in 10% glycerol-seawater onto microscope slides and visualized by either light microscopy or fluorescent microscopy with a Nikon confocal fluorescent microscope equipped with a rhodamine filter.

Results

Effects of protein kinase C modulators on larval settlement and metamorphosis

We have previously found that the juvenile hormones MF, JH I, and JH III, as well as compounds with juvenile hormone activity in insect cuticle bioassays, including arachidonic acid, are able to induce settlement and metamorphosis of *Capitella* sp. 1 metatrochophore larvae (Biggers and Laufer, 1992).

The first indication that the larvae sense the presence of JH III in the seawater is the onset of excited swimming. This response, which is typical of normal settlement behavior (Butman *et al.*, 1988; Pechenik and Cerulli, 1991), is faster than normal, with intermittent spiral-corkscrew movements, gradual body elongation, and periodic touching of the bottom of the petri dish. The larvae then settle and metamorphose into normal juvenile worms within 1 h. Both MF and JH III are very potent inducers of settlement and metamorphosis of the *Capitella* larvae at micromolar concentrations, whereas control larvae do not start to spontaneously settle and metamorphose until after 24 h (Fig. 2).

In preliminary investigations of the signal transduction process that mediates JH-induced settlement and metamorphosis of the *Capitella* larvae, we found compounds that elevate intracellular cAMP concentrations to be ineffective inducers of settlement and metamorphosis, indicating that cAMP does not act as a second messenger in this signal transduction process (Biggers and Laufer, 1992).

Based upon the ability of JH I, JH III, and arachidonic acid

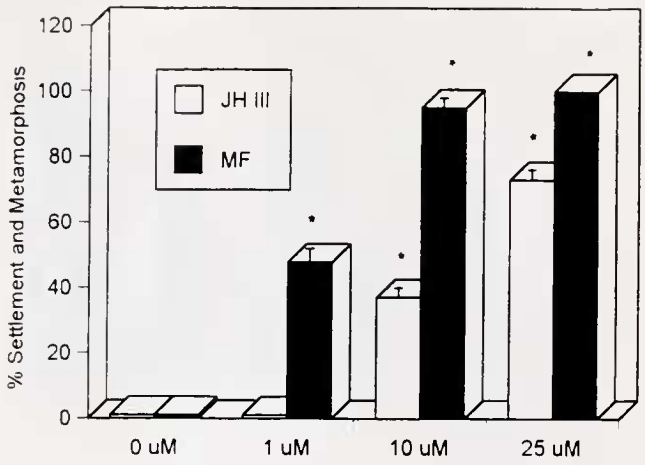

Figure 2. Concentration-dependent stimulatory effects of juvenile hormone III (JH III) and methyl farnesoate (MF) on *Capitella* larval settlement. Metatrochophore larvae, less than 1 day after release from parental brood tubes, were exposed to several concentrations of JH III or MF in seawater, ranging from 1 to 25 μM. Percent settlement and metamorphosis is shown for each concentration after 1 h. Each bar represents the percent settlement and metamorphosis for each concentration (mean $\pm$ SEM, n = 6, representing 60 larvae/concentration). Controls (0 μM) received 50 μl 95% ethanol. Asterisks indicate significant differences ($P < 0.001$, $F > 50$) between control and experimental values at each concentration tested by one-way ANOVA with a Bonferroni correction for multiple comparisons where $\alpha = 0.017$.

to activate PKC in other species including insects (Yamamoto *et al.*, 1988; Holian *et al.*, 1989; Sevala and Davey, 1989; Shearman *et al.*, 1989a; Shinomura *et al.*, 1991), and on reports that PKC activation is involved in the mediation of settlement and metamorphosis of other species of marine invertebrate larvae (Muller, 1985; Baxter and Morse, 1987; Leitz and Klingman, 1990; Morse, 1990), we further investigated the involvement of PKC activation in mediating settlement and metamorphosis of the *Capitella* larvae by testing the effects of other known PKC modulators. The phorbol ester phorbol-12,13-dibutyrate (PDBU) is a well-studied activator of PKC, and PKC has been found to be the actual cellular receptor for PDBU in some cells (Castagna *et al.*, 1982; Vandenbark *et al.*, 1984). Experiments with PDBU on the *Capitella* larvae showed that it is also a very potent inducer of settlement and metamorphosis (Fig. 3A), indicating that PKC activation is involved in mediating this process.

The effect of a PKC inhibitor was next studied to determine if it could inhibit settlement and metamorphosis induced by JH. The PKC inhibitor 1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine, abbreviated H-7 (Hidaka *et al.*, 1984), caused a concentration-dependent inhibition of *Capitella* settlement and metamorphosis induced by 25 μM JH III, when the larvae were pre-exposed to H-7 for 3 h (Fig. 3B). This result again indicates the involvement of PKC activation in mediating the effects of JH on settlement and metamorphosis.

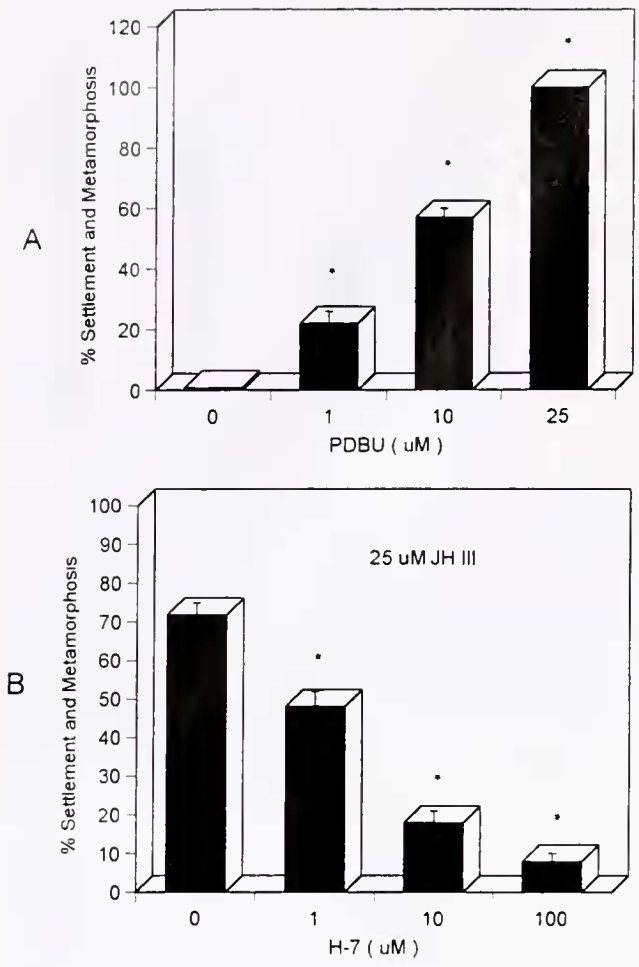

Figure 3. Effects of protein kinase C (PKC) modulators on larval settlement and metamorphosis. (A) Induction of settlement and metamorphosis by 12,13 dibutyrate (PDBU): *Capitella* metatrochophore larvae, less than 1 day after release, were exposed to the PKC activator phorbol PDBU added to the seawater at concentrations ranging from 1 to 25 μM, and settlement and metamorphosis was noted after 1 h. Controls received 50 μl 95% ethanol. Bars represent the percent settlement and metamorphosis for each concentration (mean ± SEM, $n = 6$, representing 60 larvae/concentration). Asterisks indicate significant differences ($P < 0.001$, $F > 25$) between control and experimental values at each concentration tested by one-way ANOVA with a Bonferroni correction for multiple comparisons where $\alpha = 0.017$. (B) Inhibition of juvenile hormone (JH) III-induced settlement and metamorphosis by H-7. *Capitella* metatrochophore larvae, less than 1 day after release, were pre-exposed for 3 h to concentrations of H-7, a PKC inhibitor, in seawater ranging from 1 to 100 μM, before being exposed to JH III (20 μM) for 1 h. Controls (0 μM) received 100 μl 95% ethanol followed by JH III. Bars represent the percent settlement and metamorphosis for each concentration after the 4-h time period (mean ± SEM, $n = 6$, representing 60 larvae/concentration). Asterisks same as in (A).

Activation of a protein kinase C-like enzyme in Capitella *larvae by JH-active chemicals*

Although PKC is considered to be a ubiquitous enzyme present throughout the animal kingdom and has been found in marine sponges (Muller *et al.*, 1987) and *Dictyostelium discoideum* (Jimenez *et al.*, 1989; Luderus *et al.*, 1989), its presence in polychaetes has so far not been documented. The presence of a PKC-like enzyme in *Capitella* larvae was therefore investigated, as was also the ability of JH-active compounds to activate this enzyme. In these investigations, we used an assay specific for PKC. Developed by Yasuda *et al.* (1990), this assay is based upon the specific phosphorylation by PKC of an 11-amino acid peptide sequence of myelin basic protein, which is not phosphorylated by either cAMP-dependent protein kinase (PKA), casein kinases I and II, Ca^{2+}/calmodulin-dependent protein kinase II, or phosphorylase kinase. The results demonstrate that a PKC-like enzyme does exist in *Capitella* (Fig. 4). PKC activity was linear for up to 15 min for homogenate concentrations up to 100 μg (Fig. 4A, B). The specific activity of PKC in the larval homogenates was calculated as being 6.7 fmoles ^{32}P-incorporated per minute per microgram of protein. Incubations were also done without the PKC substrate MBP_{4-14} to monitor endogenous larval protein phosphorylation, of which only a very small amount could be detected.

Experiments were next conducted to determine whether

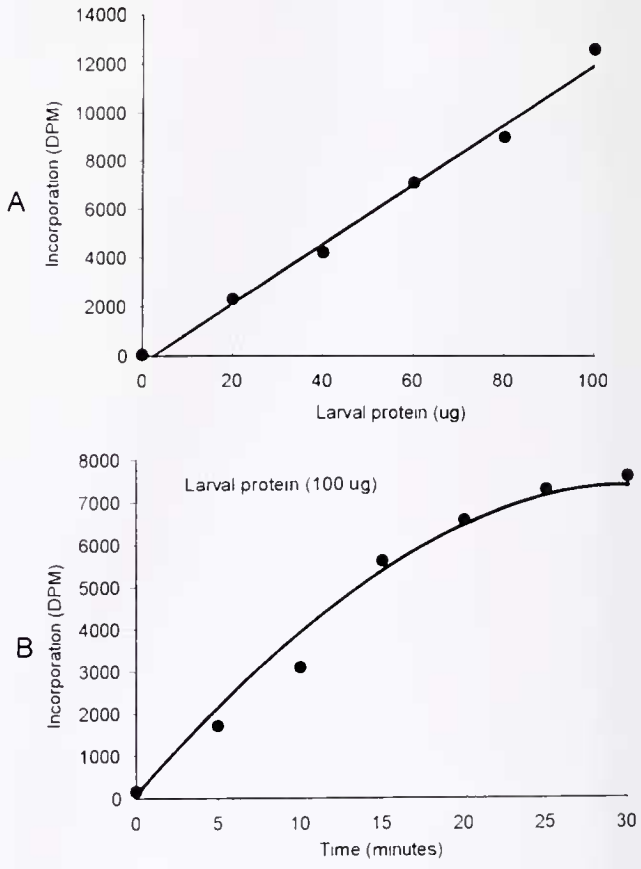

Figure 4. Protein kinase C (PKC) activity in *Capitella* larvae. PKC activity was detected in larval homogenates after activation with phosphatidylserine and diolein. ^{32}P-incorporation into MBP_{4-14} was linear with increasing protein concentrations ranging from 20 to 100 μg (A) and was also linear for 15 min using 100 μg protein (B).

the PKC-like enzyme present in the *Capitella* larvae could be activated by JH-active compounds. Incubations were carried out using 100 μg of the larval homogenates for 15 min at 30°C in the presence of either phosphatidylserine/diolein (PS/DO), 10 μM arachidonic acid (AA), 10 μM JH III, 10 μM MF, or 10 μM elaidic acid (EA). The results of this experiment indicate that JH III, MF, and AA are able to activate *Capitella* PKC *in vitro* (Fig. 5A). In comparison with activation by PS/DO, arachidonic acid was the strongest activator (94% activation), followed by MF (73% activation) and JH III (51% activation). Elaidic acid, a trans-isomer of oleic acid that does not activate rat brain PKC (Shearman *et al.*, 1989a) and does not induce settlement and metamorphosis of the *Capitella* larvae, did not activate the *Capitella* PKC. These results therefore again indicate the involvement of PKC activation in mediating settlement and metamorphosis of the *Capitella* larvae.

Since it is possible that the crude larval homogenates contain enzymes, receptors, and substrates of the diacylglycerol pathway, such as phospholipase C and guanine-binding proteins through which indirect activation of PKC may occur, we also tested whether JH could directly activate a purified preparation of rat brain PKC. Polyunsaturated fatty acids such as arachidonic acid can activate bovine brain PKC (Shearman *et al.*, 1989a) and rat brain PKC (Holian *et al.*, 1989; Shinomura *et al.*, 1991); therefore, juvenile hormones might also be able to cross species lines and activate rat brain PKC. Incubations were carried out using 5 ng purified rat brain PKC (Calbiochem Co.) in the presence of either PS/DO, 10 μM AA, 10 μM JH III, 10 μM MF, or 10 μM EA, at 37°C for 15 min. The results show that insect juvenile hormones and the crustacean juvenile hormone MF are able to directly activate rat brain PKC in the absence of phosphatidylserine and diolein (Fig. 5B). Arachidonic acid was the most potent activator tested (93% activation relative to PS/DO), whereas elaidic acid was inactive, confirming earlier work reported by Shearman *et al.* (1989a). MF caused 59% activation of the rat brain PKC and was more active than JH III (28% activation).

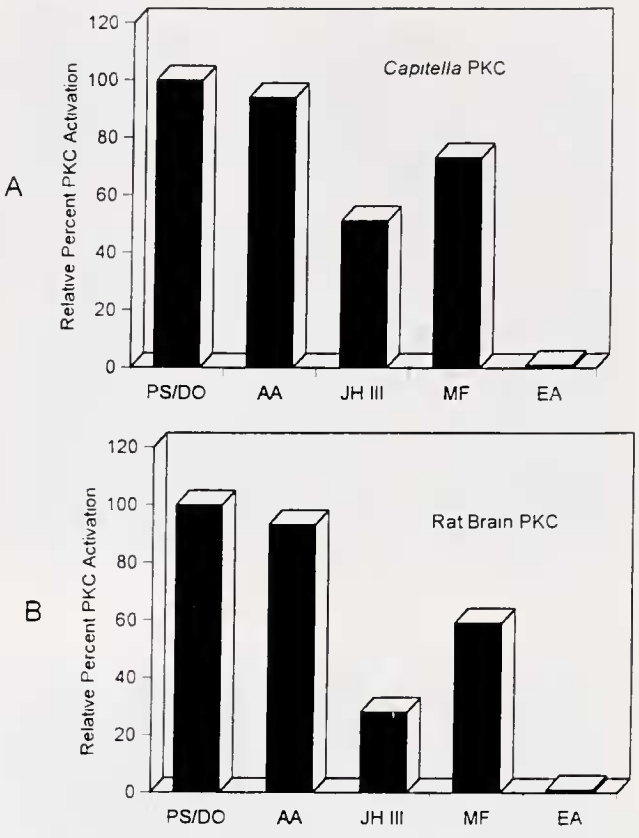

Figure 5. Activation of an enzyme resembling protein kinase C (PKC) in *Capitella* larval homogenates and purified rat brain PKC by JH-active chemicals. (A) Activation of the PKC-like enzyme present in larvae. PKC assays using *Capitella* larval homogenates were carried out in the presence of either phosphatidylserine/diolein (PS/DO), 10 μM arachidonic acid (AA), 10 μM juvenile hormone (JH) III, 10 μM *trans, trans* methyl farnesoate (MF), or 10 μM elaidic acid (EA). Incorporation of ^{32}P into MBP_{4-14} for AA, JH III, MF, and EA is shown expressed as the percentage incorporation relative to that found for the control (PS/DO). (B) Activation of purified rat brain PKC. PKC assays using 5 ng purified rat brain PKC were carried out in the presence of either phosphatidylserine/diolein (PS/DO), 10 μM arachidonic acid (AA), 10 μM JH III, 10 μM *trans, trans* methyl farnesoate (MF), or 10 μM elaidic acid (EA). Incorporation of ^{32}P into MBP_{4-14} for AA, JH III, MF, and EA is shown expressed as the percentage incorporation relative to that found for the control (PS/DO).

Localization of PKC in Capitella *by RIM-1*

To visualize the location of PKC in the *Capitella* larvae and juveniles and to identify possible chemosensory cells that would rapidly take up external chemicals or chemicals in the environment, larvae and juveniles were briefly (1 min) exposed to a fluorescently labeled protein kinase C inhibitor, rhodamine-conjugated bisindolylmaleimide (RIM-1), which has proven useful as a fluorescent probe for PKC (Chen and Poenie, 1993). After exposure to this inhibitor, the larvae or juveniles were fixed, permeabilized, rinsed to remove excess unbound RIM-1, and viewed under a fluorescent microscope. In metatrochophore larvae, distinct cells in the ciliary bands of the prototroch and telotroch and isolated cells in the apical region of the prostomium and the rest of the body displayed RIM-1 binding, indicating that these cells possess PKC (Fig. 6B). Juvenile *Capitella* exposed in the same manner displayed RIM-1 binding to cells in the apical region of the prostomium and scattered throughout the rest of the body (Fig. 6D). These results provide more evidence for the presence of a PKC-like enzyme in *Capitella* larvae and juveniles, and are consistent with a chemosensory function for apical cells in regions of the prostomium as previously noted by Eckelbarger and Grassle (1987).

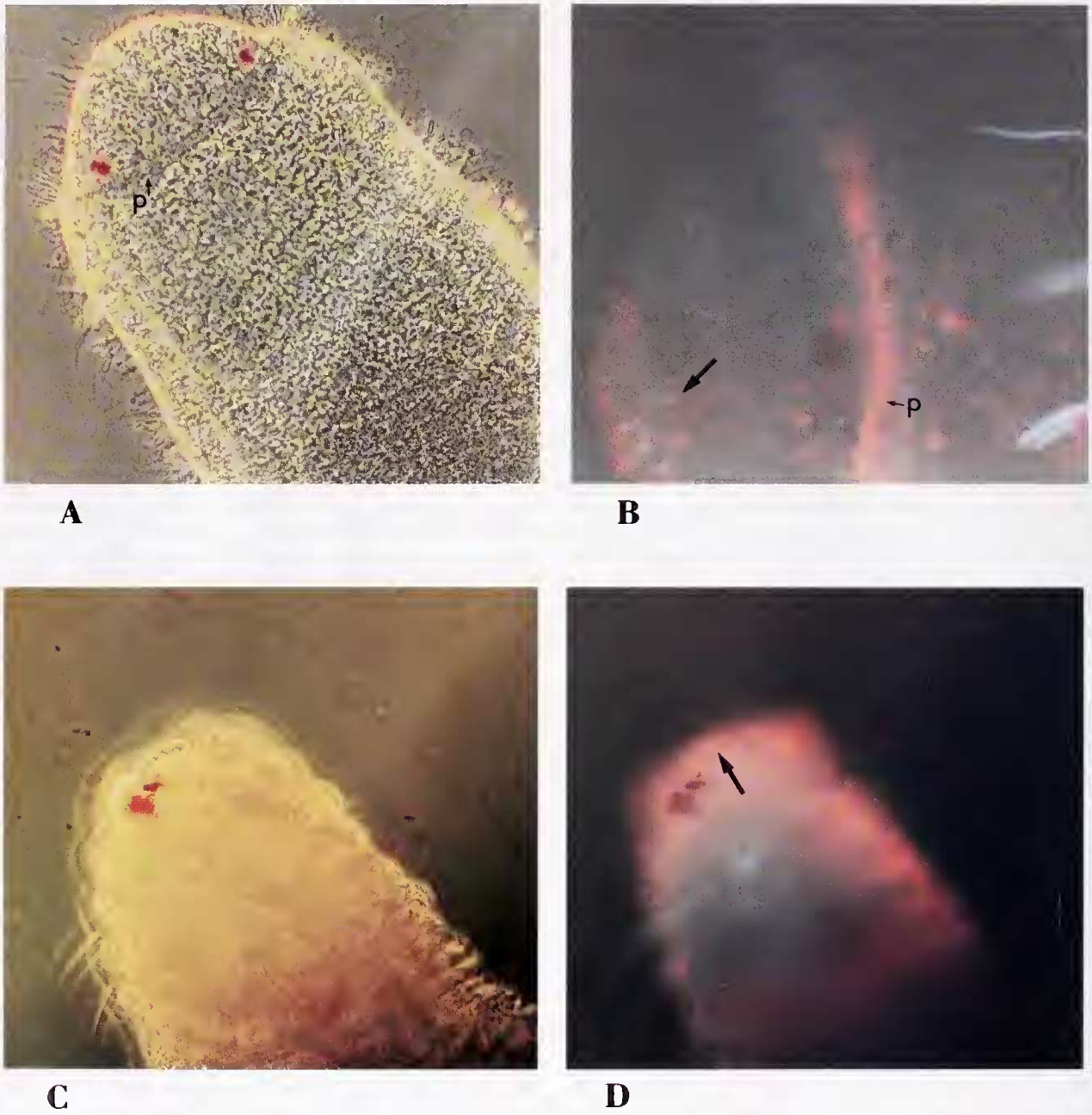

Figure 6. Localization of protein kinase C (PKC) in *Capitella* by RIM-1 analysis. Metatrochophore larvae less than 1 day after release from parental brood tubes and 1-day-old juveniles were exposed for 1 min to RIM-1, a rhodamine-conjugated PKC inhibitor. (A) Light microscopy of metatrochophore larva (400×). (B) Fluorescence microscopy of metatrochophore larva showing binding of RIM-1 to ciliary cells of the prototroch (p) as well as isolated apical cells located in the prostomium (arrow) (800×). (C) Light microscopy of juvenile *Capitella* prostomium (400×). (D) Fluorescence microscopy of juvenile *Capitella* prostomium showing binding of RIM-1 to apical cells (arrow) (400×).

Effects of potassium channel modulators

In mediating cellular responses, PKC activation has in many cases been found to result in the modulation of potassium and calcium channels (Kaczmarek, 1987; Shearman *et al.*, 1989b). The involvement of potassium channels in mediating the settlement and metamorphosis of several types of marine invertebrate larvae has also been previously demonstrated (Baloun and Morse, 1984; Yool *et al.*, 1986; Leitz and Klingman, 1990; Carpizo-Iuarte and Hadfield,

Table I

Effects of ion channel modulators on settlement and metamorphosis of Capitella *sp. I larvae*

Chemical	% Larvae settled and metamorphosed (after 1 h)
Potassium channel modulators	
KCl (20 m*M*)	100
KCl (20 m*M*) + TEA (100 m*M*)	5
4-Aminopyridine (100 μM)	100
JH III (38 μM)	100
JH III (38 μM) + nigericin (500 ng/ml)	0
Calcium channel modulators	
A23187 (400 n*M*)	100
JH III (38 μM)	100
JH III (38 μM) + $NiCl_2$ (10 m*M*)	0
JH III (38 μM) + $ZnCl_2$ (10 m*M*)	0
JH III (38 μM) + Verapamil (17 μM)	0

JH = juvenile hormone.

1998). Studies were therefore carried out to determine if the modulation of potassium channels may also mediate settlement and metamorphosis of the *Capitella* larvae. Increased external KCl concentrations in the seawater induced settlement and metamorphosis of the *Capitella* larvae in a concentration-dependent manner, with an added concentration of 20 m*M* (30 m*M* total including seawater) inducing 100% settlement and metamorphosis in 1 h (Table I). These effects of KCl appear to be mediated by K^+ ions and not Cl^- since addition of NaCl had no effect on settlement and metamorphosis. The effect of tetraethylammonium chloride (TEA), a known blocker of potassium currents, was next studied to determine its effect on settlement and metamorphosis. TEA did not induce settlement and metamorphosis of the *Capitella* larvae, but instead inhibited the stimulatory response of the larvae to KCl, with a concentration of 100 m*M* TEA almost completely inhibiting settlement and metamorphosis (Table I). Settlement and metamorphosis of the *Capitella* larvae was, however, stimulated in a concentration-dependent manner by 4-aminopyridine (4-AP), another potassium channel blocker, which blocks outward rectifying potassium currents. A concentration of 100 μM 4-AP stimulated 100% settlement and metamorphosis of the larvae within 1 h (Table I). The effects of the potassium channel ionophore, nigericin, was next studied. Pre-exposure of the larvae to nigericin for 30 min inhibited the response of the larvae to JH III in a concentration-dependent manner, with 500 ng/ml nigericin causing 100% inhibition of settlement and metamorphosis in response to JH III in 1 h (Table I).

Effects of calcium channel modulators

Calcium channels are in many cases activated in response to membrane depolarization and in response to PKC activators (Kaczmarek, 1987; Shearman *et al.*, 1989b). Calcium channel modulators were therefore studied for their effects on settlement and metamorphosis of the *Capitella* larvae. Pre-exposure of the larvae to the known calcium channel blockers Ni^{2+} at a concentration of 10 m*M*, Zn^{2+} at a concentration of 10 m*M*, and verapamil at a concentration of 17 μM completely inhibited the settlement- and metamorphosis-inducing effects of JH III (Table I). Next, to determine whether an influx of calcium could stimulate settlement and metamorphosis, the effect of the calcium channel ionophore A23187 was tested. A23187 proved to have a potent, concentration-dependent effect on larval settlement and metamorphosis (Fig. 7), with a concentration of 400 n*M* stimulating 100% of the larvae to settle and metamorphose in 1 h (Table I). These results therefore suggest that JH III activation of PKC may lead to the opening of calcium channels.

Discussion

The ability of juvenile hormones and compounds with JH activity to induce settlement and metamorphosis in *Capitella* larvae raises the possibility that these compounds may act on the larvae through a mechanism similar to that by which they affect insect metamorphosis and reproduction. In insects, juvenile hormones have multiple mechanisms for regulating metamorphosis and reproduction. For example, they may act through nuclear receptors and transcriptional regulation (Jones *et al.*, 1993; Palli *et al.*, 1994; Jones and

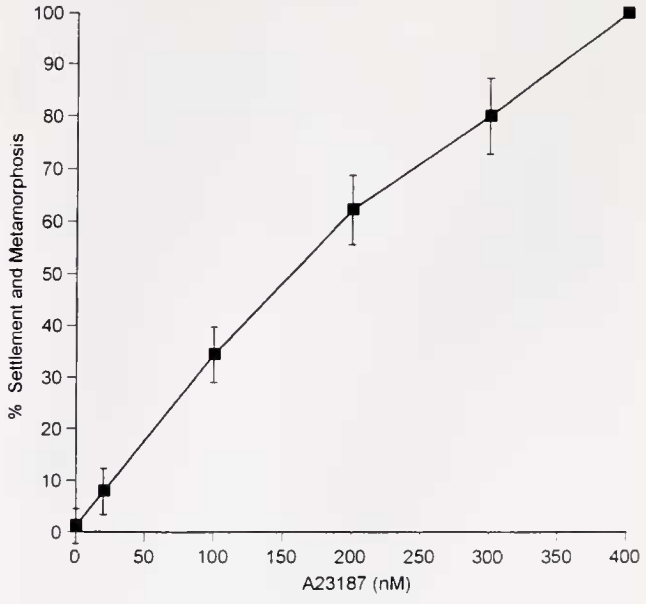

Figure 7. Effect of the calcium ionophore A23187 on settlement and metamorphosis of *Capitella* larvae. Metatrochophore larvae less than 1 day after release, exposed for 1 h to concentrations of A23187 ranging from 25 to 400 n*M* in seawater, were stimulated to settle and metamorphose in a concentration-dependent manner.

Sharp, 1997), or by affecting mRNA stability (Jones *et al.*, 1993) and mRNA translation (Ilan *et al.*, 1972). JH I affects follicle cell patency in *Rhodnius prolixus* through—another well-studied mechanism (Sevala and Davey, 1989)—a signal transduction cascade involving a membrane receptor, activation of PKC, and the subsequent activation of a Na/K-ATPase. Other studies (Yamamoto *et al.*, 1988) have demonstrated that PKC activation and opening of calcium channels is involved in the mechanism by which JH III induces protein synthesis in *Drosophila* male accessory glands. Our studies indicate that PKC activation and ion channel modulation are also involved in the process of chemosensory signal transduction by which JH and compounds with JH activity induce settlement and metamorphosis of larvae of *Capitella* sp. I.

In addition to JH I and JH III, other known activators of PKC can also induce settlement of *Capitella* larvae. One example is arachidonic acid, which has shown JH activity in insect cuticle bioassays (Slama, 1962) and strongly activates PKC from bovine brain (Shearman *et al.*, 1989a) and rat brain (Holian *et al.*, 1989; Shinomura *et al.*, 1991). The potency of the phorbol ester PDBU in inducing settling and metamorphosis of *Capitella* larvae (Fig. 3A) is particularly good evidence for the activation of a protein kinase C-like enzyme, since it is well established that PDBU directly activates PKC in mammalian tissues (Castagna *et al.*, 1982; Nishizuka, 1984; Vandenbark *et al.*, 1984; Parker *et al.*, 1986). The PKC inhibitor H-7 is able to inhibit the settlement and metamorphosis effects of JH III (Fig. 3B). Although H-7 at higher concentrations also inhibits other protein kinases such as cyclic AMP-dependent protein kinase (PKA) (Hidaka *et al.*, 1984), the effects of H-7 on the *Capitella* larvae are probably due to the inhibition of PKC and not PKA, since PDBU, which induces settlement and metamorphosis, activates only PKC and not PKA (Castagna *et al.*, 1982).

PKC is regarded as a ubiquitous enzyme present throughout the animal kingdom (Nishizuka, 1984), having been demonstrated in *Dictyostelium* (Luderus *et al.*, 1989; Jimenez *et al.*, 1989) and sponges (Muller *et al.*, 1987) as well as in mammalian tissues. Other studies have also demonstrated that PKC activation is involved in the signal transduction processes that mediate settlement and metamorphosis of marine invertebrate larvae of the coelenterate *Hydractinia* (Muller, 1985; Leitz and Klingman, 1990) and the mollusc *Haliotis rufescens* (Baxter and Morse, 1987; Morse, 1990). It is therefore likely that *Capitella* also possesses a PKC-like enzyme that is involved in mediating larval settlement and metamorphosis. Our data indicate that *Capitella* does possess such an enzyme, since crude homogenates of the larvae show PKC activity in a selective PKC assay (Fig. 4). More studies are needed to further characterize the enzyme and determine its requirement for calcium.

JH appears to activate PKC in the *Capitella* larvae directly, much like the mechanism of action of phorbol esters. Our results show that MF, JH III, and arachidonic acid can, *in vitro*, directly activate the PKC-like enzyme in *Capitella* as well as purified PKC from rat brain in the complete absence of the normal membrane inducers phosphatidyl serine and diacylglycerol (Fig. 5). These data therefore indicate that juvenile hormones, as well as JH-active compounds such as arachidonic acid, are able to bind to the lipid-binding site in PKC (Parker *et al.*, 1986; Ziesel, 1993). This is perhaps not surprising given that phorbol esters and juvenile hormones are both terpenoid compounds: phorbol esters are diterpenoids and juvenile hormones are sesquiterpenoids.

In sensing JH-active compounds in the seawater, these lipophilic compounds presumably pass through the membrane of ciliary epithelial chemosensory cells similar to those reported to transduce chemical signals for settlement and metamorphosis in larvae of the abalone *Haliotis* (Trapido-Rosenthal and Morse, 1986), the cnidarian *Hydractinia* (Schwoerer-Bohning *et al.*, 1990), the polychaete *Phragmatopoma californica* (Amieva *et al.*, 1987), and the sea star *Luidia senegalensis* (Komatsu *et al.*, 1991). Our studies with the fluorescent PKC inhibitor RIM-1 provide more evidence that *Capitella* larvae possess a PKC-like enzyme and that PKC is present in chemosensory cells. The RIM-1 presumably was able to directly enter chemosensory cells of the larvae that allow rapid uptake of external chemicals, since the larvae were exposed to RIM-1 only briefly (1 min). The intense RIM-1 binding in isolated cells of the prostomium (Fig. 6) may represent the presence of PKC in apical cilia that are thought, on the basis of evidence from electron microscopy (Eckelbarger and Grassle, 1987) to serve a chemosensory function. However, entry of RIM-1 into the larvae through other processes cannot be ruled out, since RIM-1 binding was found in cells throughout the body, especially in ciliary cells of the prototroch and telotroch, and in both metatrochophore larvae and juveniles.

It is likely that JH-active compounds, after passing through the membrane of chemosensory cells, bind with an inactive PKC present in the cytosol, which, as in other species (Nishizuka, 1984), then becomes active and is translocated to the membrane. It is evident from our studies that micromolar concentrations of JH-active compounds are able to activate PKC and thereby induce settlement and metamorphosis. Concentrations of 10 μM JH III, MF, and arachidonic acid activate both *Capitella* PKC and rat brain PKC (Fig. 4), and the same concentrations of these chemicals dissolved in the seawater also induce settlement and metamorphosis of the larvae. The inability of elaidic acid to activate PKC *in vitro* and to induce settlement and metamorphosis is further evidence that PKC activation is involved in mediating settlement and metamorphosis of the *Capitella* larvae.

In *Capitella* larvae, PKC activation may cause several

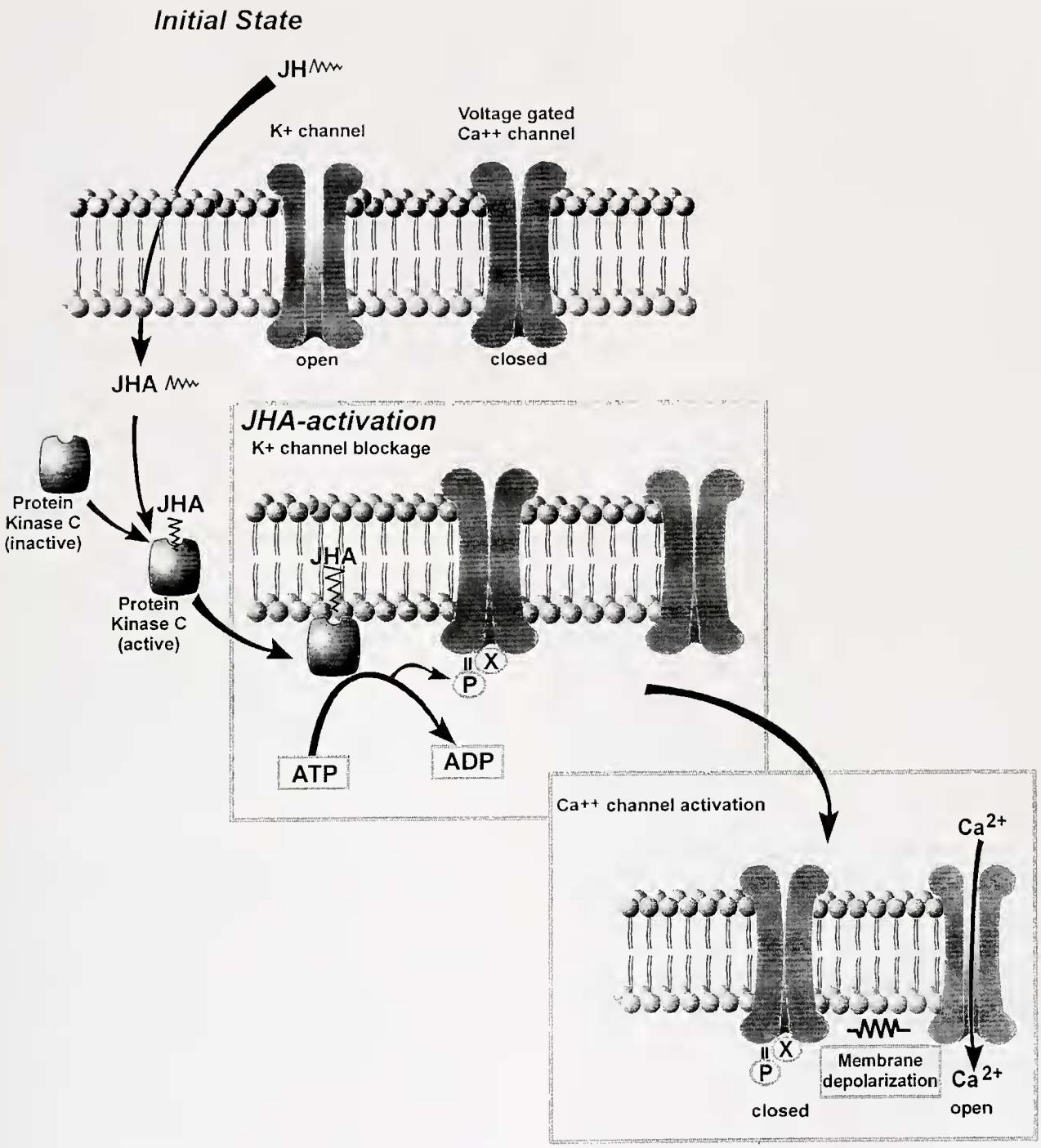

Figure 8. Model for the juvenile hormone (JH) signal-transduction pathway in *Capitella* sp. I larvae. In this proposed model, JH induces settlement and metamorphosis by directly interacting with and activating cytosolic protein kinase C (PKC), which acts as the cellular receptor in the larval chemosensory cells. This activation of PKC then causes the closure of potassium channels by phosphorylation, invoking membrane depolarization, and the subsequent opening of voltage-activated calcium channels. PKC and calcium may in subsequent steps activate transcription factors and protein kinases, leading to changes in gene activity, and also cause neurotransmitter release.

cellular events that transduce the external JH signal and lead to settlement and metamorphosis. One well-characterized effect of PKC activation is the modulation of ion transporters such as a Na+/K+ ATPase (Ilenchuk and Davey, 1987; Sevala and Davey, 1989), Na+/H+ exchangers (Berridge, 1984), and ion channels (Kaczmarek, 1987; Shearman *et al.*,

1989b). The modulation of potassium channels, resulting in membrane depolarization and neural relay of external chemical settlement cues, has been demonstrated to be involved with the settlement and metamorphosis of several types of marine invertebrate larvae, including those of the abalone *Haliotis rufescens* (Baloun and Morse, 1984), the nudibranch *Phestilla sibogae* and the prosobranch *Astraea undosa* (Yool *et al.*, 1986), the cnidarian *Hydractinia* (Leitz and Klingman, 1990), and the polychaetes *Phragmatopoma californica* (Yool *et al.*, 1986), and *Hydroides elegans* (Carpizo-Ituarte and Hadfield, 1998). Pheromone reception by insects and non-insect species has also in some instances been demonstrated to be mediated by PKC activation and the concommitant modulation of ion channel activity (Zufall and Hatt, 1991; Stengl, 1993). Like those studies, our investigations suggest that ion channel modulation is also involved. We found that the addition of excess KCl to the seawater stimulated settlement and metamorphosis, whereas this effect was negated by simultaneous addition of the potassium channel blocker tetraethylammonium chloride (TEA). These results indicate that TEA blocks settlement and metamorphosis by preventing the entry of excess potassium. These results are similar to those reported by Carpizo-Ituarte and Hadfield (1998) in their investigations with larvae of the polychaete *Hydroides elegans*. TEA did not induce settlement and metamorphosis of the *Capitella* larvae at the concentrations tested; however, the larvae were induced to settle and metamophose in response to another potassium channel blocker, 4-aminopyridine (4-AP), which causes blockage of outward rectifying potassium currents (Alkon *et al.*, 1986). These data indicate that blockage of outward rectifying potassium channels can induce settlement and metamorphosis of the *Capitella* larvae. Since studies by Leitz and Klingman (1990) demonstrated that potassium channel closure was involved in mediating settlement and metamorphosis of *Hydractinia* larvae in response to PKC-activating diacylglycerols, we tested the possibility that JH induces settlement of the *Capitella* larvae through closure of potassium channels in response to PKC activation. Our findings that the potassium channel ionophore nigericin inhibits settlement and metamorphosis induced by JH III and that 4-AP can directly stimulate settlement and metamorphosis provide evidence that JH stimulates settlement and metamorphosis through the closure of potassium channels.

Since potassium channel closure may cause membrane depolarization, it is possible that voltage-gated calcium channels are activated during this process and participate in mediating settlement and metamorphosis. Our studies support this idea, since the calcium channel blockers Ni^{2+}, Zn^{2+}, and verapamil inhibited settlement and metamorphosis induced by JH III, whereas the calcium channel ionophore A23187 induced settlement and metamorphosis.

Our interpretation of these data is that juvenile hormones and JH-active chemicals induce settlement and metamorphosis of *Capitella* larvae through activation of PKC, which causes closure of potassium channels; the reduced efflux of potassium depolarizes the membranes of the chemoreceptor cells, leading to the opening of voltage-activated calcium channels (Fig. 8). In eliciting metamorphosis, PKC activation by JH may activate transcription factors such as nuclear factor-kB (NF-kB) or stimulate the mitogen-activated protein kinase (MAP-kinase) pathway. Membrane depolarization and calcium influx in PC12 cells has been demonstrated to activate the MAP kinase pathway (Rosen *et al.*, 1994; Rosen and Greenberg, 1996). PKC modulation also plays a role in regulating neural differentiation of *Xenopus* (Durston and Otte, 1991), epithelial patterning in *Hydra* (Shenk and Steele, 1993), and skin morphogenesis in chickens (Noveen *et al.*, 1995). The molecular mechanisms by which PKC and calcium may be regulating settlement and metamorphosis of the *Capitella* larvae are now the subject of our ongoing research.

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